B.TECH FINAL YEAR THESIS ON

BIODEGRADATION OF PETROLEUM HYDROCARBONS IN SOIL BY MICROBIAL CONSORTIUM

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107CH025

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CERTIFICATE

This is to certify that the seminar report on **Biodegradation of Petroleum Hydrocarbons in Soil by Microbial Consortium** submitted by **Somya Vashishtha** to National Institute of Technology, Rourkela under my supervision and is worthy for the fulfillment of the degree of Bachelor of Technology (Chemical Engineering) of the Institute. The candidate has fulfilled all prescribed requirements and the thesis, which is based on candidate's own work, has not been submitted elsewhere.

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Thanking you,

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Abstract

The most common problems today are caused due to petroleum products, their use and their degradation. If exposed to the environment they can be hazardous to the surroundings as well as life forms. Thus remediation of these is of utmost importance. Bioremediation is a non-invasive and cost effective technique for the clean-up of these petroleum hydrocarbons.

In this study we have investigated the ability of microorganisms present in the soil itself to degrade these hydrocarbons so that contaminated soils can be treated using microbial consortium. Soil from areas near workhouses and garages is used to isolate the microorganism which is further used to create an inoculum, whose ability to degrade heavy as well as light crude oil is assessed quantitatively. The quantitative analysis is carried out gravimetrically. The process parameters are optimized first and then degradation study is carried out in terms of biomass, dissolved oxygen and the amount of oil degraded.

Keywords: Biodegradation, Petroleum hydrocarbons, bioremediation

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CHAPTER 1

INTRODUCTION

1. Introduction:

Oil spills are a common problem these days. It is the release of liquid petroleum hydrocarbons into the environment; generally it refers to the marine oil spills. They may be caused by natural disasters like earthquakes in the sea surface or due to accidental leaks during exploration, refining, storage and transportation. The causes can be numerous but the consequences are the same. Spilled oil can be crude oil or even refined products. In case of refined products it is difficult to clean up because the fractions contain many long chain or aromatic hydrocarbons, which are difficult to degrade. In case of crude oil too, the different types such as heavy or light crude oil can affect the clean-up procedures. Crude oil spreads very rapidly on the sea surface and after a short period of time the thickness of the oil film can be as low as 1mm. It is therefore necessary to prevent the spreading and stop it from reaching the shoreline, as once it reaches the shoreline it can seep into the groundwater and contaminate it. Also contamination of the soil can take place in turn affecting the marine ecosystem in the region; it can spread over the plumage of the birds making them more susceptible to temperature fluctuations and also less buoyant on water. It can also cause kidney and liver damage and irritation of the digestive tract, if ingested. Marine animals like sea otter and seals when exposed to oil; can reduce their insulation abilities causing hypothermia and body temperature fluctuations. As there is a layer of oil on the top, less sunlight reaches the marine flora and phytoplankton, reducing their photosynthesis. All of this can damage the food chain and hence the ecosystem of the region. The effects of oil spill are hence widespread and such that can be apparent for a long period of time.

Some of the largest oil spills have taken place all over the world; Iran, Iraq, Persian Gulf, Uzbekistan, Kuwait, United States, Gulf of Mexico, Pakistan and even India. The most recent oil spill was the spill off the Mumbai Coast in January 2011; many such examples can be cited like the spill in August 2010 caused due to seeping oil from a sinking ship. The most

recent case of oil spill in Orissa was the one on Gopalpur beach in April 2010 causing the death of many Ridley turtles that breed there during that period. The nation's coastal waters are being exploited at an alarming rate which has had severe impacts on much of India's marine biodiversity as well as the livelihoods of coastal fishing communities. It hence becomes a necessity to take steps in this regard in order to save the marine life.

Many mechanical and chemical recovery methods can be applied for clean-up depending on the type of oil spilled, temperature of the water, type of shoreline involved etc. Some of these methods are controlled burning, use of dispersants, dredging, skimming, solidifying, vacuuming and centrifuging etc. All these methods are viable but require lot of equipments and chemicals which is not very economically viable. Also these methods can be considered as artificial i.e. in turn may cause some harm to the environment. Bioremediation is a method where microorganisms are used for the clean-up processes. They not only clean-up the area but their biomass also replaces other populations in the food chain. A number of microorganisms have been found to have the capability to degrade the hydrocarbons from the oil. These include strains of bacteria, fungi, yeast, algae etc.

Spilled oil can also contaminate the soil in the area. Bioremediation for the soil requires the identification of microbes that are present in the soil itself so that in case of a large spill these can be stimulated further in order to clean-up the area. Identification of such strains can ensure better efficiency of remediation as these strains will be well adapted to grow in the soil system. My objective is to hence isolate a strain of such oil degrading microorganism so that it can be cultured under optimum conditions and used as a remediation tool.

CHAPTER 2

LITERATURE REVIEW

2. Literature Review

Oil type, weather, wind and wave conditions, as well as air and sea temperature, all play important roles in ultimate fate of spilled oil in marine environment. After oil is discharged in the environment, a wide variety of physical, chemical and biological processes begin to transform the discharged oil. These chemical and physical processes are collectively called weathering and act to change the composition, behaviour, route of exposure and toxicity of discharged oil. There are ten weathering processes discussed as below:

- Spreading and Advection: Spreading is the movement of the entire oil slick horizontally on the surface of water due to effects of gravity, inertia, friction, viscosity and surface tension. On calm water spreading occurs in a circular pattern outward from the centre of release point. Advection is the movement of oil due to overlying winds or underlying currents. They increase the surface area of the oil, thereby increasing its exposure to air, sunlight and underlying water. They are not uniform and do not affect the chemical composition of the oil. Spreading dominates the initial stages of the spill and involves the whole oil. Spreading and advection occur immediately following the release and continue for approximately one week to ten days for large slicks, or until it is contained by the shoreline, collection efforts or any other obstruction.
- Evaporation: Evaporation is the preferential transfer of light and medium weight components of the oil from the liquid phase to the vapour phase. The chemical composition of the slick is physically altered and although the volume decreases, the remaining components have greater viscosity and specific gravity leading to thickening of the slick and formation of tar balls, tar masts etc. It also affects the oil's toxicity; the lighter components which are considered more toxic undergo evaporation and reduce the toxicity of the oil. It starts immediately after discharge and continues

upto approximately two weeks. Majority of the evaporation occurs in first 12 hours with the lighter components evaporating at a faster rate and disappearing within 48 to 72 hours. The rate of evaporation is greatly reduced after the first week and the heavier components remain in the slick.

- Dissolution: Dissolution is the transfer of oil components (not the whole) from the slick on the surface into the solution in the water column. It is relatively insignificant in the overall weathering process in terms of reduction of volume, because many of the same components that would dissolve would typically evaporate first. Even when components do dissolve, they are removed by subsequent evaporation or other weathering processes such as biodegradation or photo-oxidation. Once dissolved these components are bioavailable and if exposed to marine life can cause environmental impacts and injuries. The largest concentration is found near the surface or the release point and hence the effect on marine life is fairly localised and short lived. It occurs within 24 hours of a spill.
- Natural Dispersion: Natural Dispersion is the process of forming small oil droplets that become incorporated in the water column in the form of a dilute oil-in-water suspension. It occurs when breaking waves mix the oil in the water column, as soon as the oil spills and reaches a maximum rate within 10 hours following the spill but may continue for several weeks; within 100 hours it overtakes spreading as the primary mechanism for transport of oil from the spill site. It reduces the volume at the slick surface but does not change the physicochemical properties of the oil. After evaporation it is the most important process in the disappearance of the slick, it is estimated to range from 10 to 60% per day for first three days of the spill, depending on the sea state but independent of the oil type.

- Emulsification: Emulsification is the mixing of sea water droplets into oil spilled on water's surface forming a water in oil emulsion. Majority of the weathering processes can be slowed once a stable emulsion is formed, also it greatly increases the volume. It begins during the first day of the spill and can continue to occur throughout the first year. The largest volume is typically formed in the first week after the loss of lighter weight components due to evaporation and dissolution.
- Photo-Oxidation: Photo-oxidation is when sunlight (ultraviolet and near ultraviolet regions of the spectrum) in the presence of oxygen transforms hydrocarbons by photo oxygenation (increasing the oxygen content of a hydrocarbon) into new by-products. The ultimate fate of these by-products removal to and dissipation into the atmosphere and water column. It results in changing in the interfacial properties of the oil, affecting spreading and emulsion formation, and may result in transfer of toxic by-products into the water column due to the by-product's enhanced water solubility. It is relatively insignificant in the overall weathering process as it is interrupted by nights and is lessened on cloudy days. It does not peak at any particular time as it depends on light intensity, duration, weather conditions and extent of emulsification. It can start within several hours of a spill and can last for several weeks to a month.
- Sedimentation and Shoreline Stranding: Sedimentation is the incorporation of oil within both bottom and suspended sediments. Sedimentation can be caused due to a number of reasons; it may be due to adherence of oil to detrital particles or due to undigested oil droplets passing in the fecal matter of marine organisms. Shoreline stranding is the visible accumulation of petroleum along the water's edge (shoreline) following a spill. It is affected by proximity of the spill to the affected shoreline, intensity of current and wave action on the affected shoreline and persistence of the spilled product. Sedimentation can begin immediately after the spill but increases and

peaks after several weeks, whereas shoreline stranding is a function of the distance of the shoreline from the spill and chemical nature of spilled oil.

- Biodegradation: Biodegradation is the process where naturally occurring bacteria and fungi consume hydrocarbons to use as a food source, giving out water and carbondioxide as waste products. It occurs on the water surface, in the water column, in the sediments and at the shoreline. Microbes which can use hydrocarbons occur naturally, but multiply further following a spill as additional food sources become available. It is a slow process and is dominant only after the components toxic to the microbes have weathered below a threshold value by other physical processes. It is dominant for light and medium weight components, heavy weight components are too structurally complex for the microbes to degrade right away, they may be degraded after several months or even years. It can begin immediately following a spill and can continue as long as degradable hydrocarbons are present. It peaks within the first month of the spill but can eventually be limited by the availability of nutrients in the environment for their growth. If nutrients are added then the microbes can grow along the shoreline degrading at a faster rate [1].

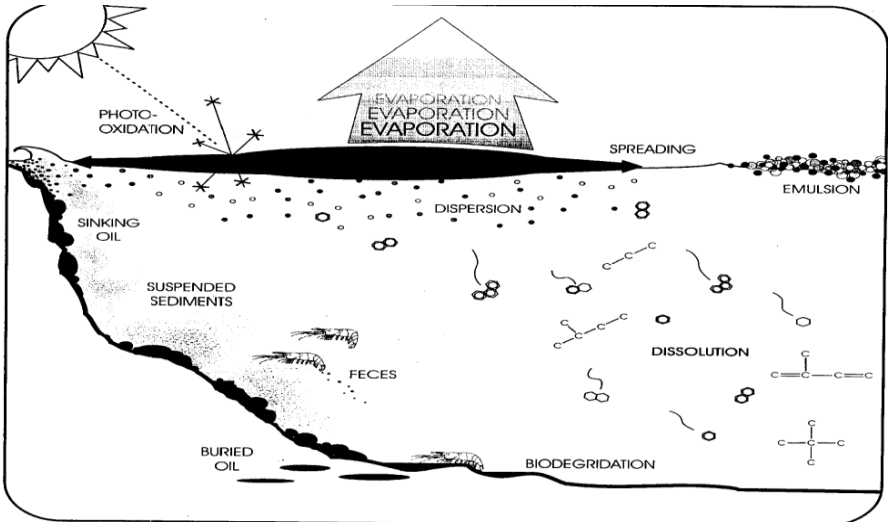

Figure 1: Summary of all the weathering processes [1]

Weathering processes occur simultaneously, one does not stop before the other begins. The order in which these processes presented is instantaneous, the relative significance of these processes may change if the spill occurred below the water surface or in tropical or ice conditions. The spill chronology may also vary if the spill is near the shoreline in which case it can contaminate the soil and groundwater even before the weathering or clean up processes start. Also there are many onshore and offshore operations in a petroleum industry that can cause soil pollution and aquifer contamination. Aquifer contamination can also take place because of the migration of the oil through the porous media and its subsequent adsorption on the rock surface. Many technologies have been proposed for the treatment of oil contaminated sites; these can be performed by two basic processes in-situ and ex-situ treatment using different cleaning technologies such as thermal treatment, biological treatment, chemical extraction and soil washing, and aeration accumulation techniques.

In-situ treatment methods: These methods require that the contaminated subsoil must be excavated or scraped. Treatment is carried out in subsoil either by biological means such as oil degradation by microorganisms, or chemical physical processes such as incineration, air sparging, and soil air suction extraction or through combinations of the two processes, depending on the spectrum of contaminants. To conduct this treatment vertical and horizontal drilling processes may be required. Also it is more effective on sandy soils than in soils that contain clay. Bioremediation can be applied with the help of spreading units in case the contamination is at the surface. There are a lot of factors like availability of nutrients and oxygen, the bioavailability of the contaminants, the penetration of the contamination, the type of soil, saturation of groundwater etc. that affect the selection of bioremediation as a clean-up process. It is suitable to treat contaminated sites of petrol stations and refinery sites and to those areas where the water table is close to the surface.

Air sparging or soil venting can be applied to extract contaminants from the soil as well as groundwater saturated soil by mobilization of the volatile component. It also accelerated the growth of aerobic bacteria in the contaminated area by oxygen feeding. Slurping is a process in which special well completion is required to extract the oil by means of vacuum. It is preferred if the contamination is in the boundary area between groundwater saturated soil and groundwater unsaturated soil. Soil air suction is also a method where suction extraction of contaminated soil air is done by means of vacuum to remove volatile hydrocarbon from contaminated area. In-situ steam injection is another method where steam at high temperature and compressed air are injected into the contaminated soil. The temperature of the steam should be greater than the boiling points of volatile components. With this process the contaminants can be converted to gaseous or volatile phase and the air, vapour and the released hydrocarbon compounds can be removed by extraction wells.

Ex-situ Treatment Methods: For these methods soil must be removed to an off site remediation facility. The treatment is carried out by incinerating the soil, chemical extraction or soil washing to remove the hydrocarbon components. The important methods of ex-situ treatment are steam stripping and combustion, extraction methods and biological methods. The chemical extraction method involves the usage of different solvents, where the contaminants are dissociated from the soil, dissolved or suspended in the solvents. It can be applied for different types of crude and soils but is more effective for soil with low clay content. It can be performed in-situ or ex-situ, but in ex-situ the clean-up period is relatively short and the efficiency is better controlled. Surfactant washing too can be applied in-situ or ex-situ. Ex-situ methods can be applied only if the amount of contaminated soil is small or if the contamination has occurred in a residential or industrial area where in-situ treatment cannot be applied. Ex-situ treatment leads to a higher degree of remediation because of controllability of many factors like pH, temperature, salinity, moisture etc. [2].

It is evident from the weathering processes that biodegradation is a natural process that can be easily manipulated and controlled quite economically. When biodegradation is altered or enhanced manually it is known as bioremediation. It can be defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities [3].

The advantages of this process can be listed as follows:

- It usually involves only minimal physical disruption of the site.
- It causes no or only minor and short lived adverse effects when used correctly.
- It may remove the some of the toxic components from a spill site more quickly than evaporation alone.
- It is accomplished on site and offers a simpler and thorough solution to the polluted areas than the mechanical methods.
- Bioremediation equipment and logistics are usually simpler and less labour intensive, hence costing less than other technologies.

Like every process it also has a few disadvantages like:

- If there is a large off shore spill then bioremediation may not be appropriate as the initial defensive measure as it requires longer time for application.
- It has to be specifically tailored according to each polluted site depending on the type of soil and the spectrum of contaminants [4].

It is evident that the merits outweigh the demerits for the process and hence it is only beneficial to adopt bioremediation as a clean-up technology. There are two main approaches to oil spill bioremediation:

1. Bioaugmentation, in which known oil degrading bacteria are added to supplement the original microbial population.

2. Biostimulation, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth limiting cosubstrates[3].

2.1 Microbial Degradation of petroleum Hydrocarbons

Biodegradation of petroleum hydrocarbons is a complex process that depends on the nature and amount of hydrocarbons present. Petroleum hydrocarbon compounds bind to soil components, and they are difficult to be removed or degraded. Hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all. The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions[3].

A number of authors have extracted various microorganisms from soils of different environments which have been shown to degrade different hydrocarbons[3]. Some of the examples are listed as follows:

- The recognition of biodegraded petroleum-derived aromatic hydrocarbons in marine sediments was reported by Jones et al.[5]. They studied the extensive biodegradation of alkyl aromatics in marine sediments which occurred prior to detectable biodegradation of n-alkane profile of the crude oil and the microorganisms, namely, *Arthrobacter, Burkholderia, Mycobacterium, Pseudomonas, Sphingomonas, and Rhodococcus* were found to be involved for alkylaromatic degradation.
- Microbial degradation of petroleum hydrocarbons in a polluted tropical stream in Lagos, Nigeria was reported by Adebusoye et al. [6]. Nine bacterial strains, namely, *Pseudomonas fluorescens, P. aeruginosa, Bacillus subtilis, Bacillus* sp., *Alcaligenes* sp., *Acinetobacter lwoffi, Flavobacterium sp., Micrococcus roseus, and*

Corynebacterium sp. were isolated from the polluted stream which could degrade crude oil.

- Bacteria are the most active agents in petroleum degradation, and they work as primary degraders of spilled oil in environment [7,8]. Floodgate [9] listed 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi which were isolated from marine environment. A similar compilation by Bartha and Bossert [10] included 22 genera of bacteria and 31 genera of fungi.
- Crude petroleum oil from petroleum contaminated soil from North East India was reported by Das and Mukherjee [11]. *Acinetobacter* sp. was found to be capable of utilizing n-alkanes of chain length C_{10}–C_{40} as a sole source of carbon. Bacterial genera, namely, *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia,* and *Mycobacterium* isolated from petroleum contaminated soil proved to be the potential organisms for hydrocarbon degradation .
- Fungal genera, namely, *Amorphoteca*, *Neosartorya*, *Talaromyces,* and *Graphium* and yeast genera, namely, *Candida, Yarrowia,* and *Pichia* were isolated from petroleum contaminated soil and proved to be the potential organisms for hydrocarbon degradation [12]. Singh [13] also reported a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium,* and *Pencillium* which were also found to be the potential degrader of crude oil hydrocarbons.
- The yeast species, namely, *Candida lipolytica, Rhodotorula mucilaginosa, Geotrichum* sp*, and Trichosporon mucoides* isolated from contaminated water were noted to degrade petroleum compounds[14].
- Walker et al. [15] isolated an alga, *Prototheca zopfi* which was capable of utilizing crude oil and a mixed hydrocarbon substrate and exhibited extensive degradation of n-alkanes and isoalkanes as well as aromatic hydrocarbons.

- Cerniglia et al. [16] observed that nine cyanobacteria, five green algae, one red alga, one brown alga, and two diatoms could oxidize naphthalene. Protozoa, by contrast, had not been shown to utilize hydrocarbons.

2.2 Mechanism of Petroleum Hydrocarbon Degradation

The mechanism of degradation can be briefly summarised in the following figure:

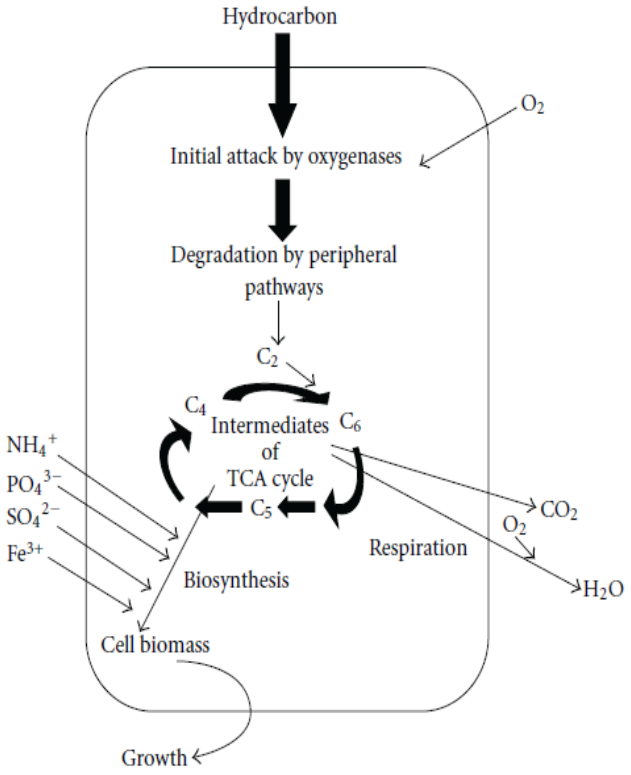

Figure 2: Mechanism of Hydrocarbon Degradation [3]

The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate, pyruvate. Sugars required for various biosyntheses and

growth are synthesized by gluconeogenesis. The degradation of petroleum hydrocarbons can be mediated by specific enzyme system. Other mechanisms involved are (1) attachment of microbial cell to substrates and (2) production of biosurfactants. Biosurfactants are heterogenous group of surface active chemical compounds produced by a variety of microorganisms. Surfactants enhance solubilisation and removal of contaminants. Biodegradation is also enhance by biosurfactants due to increased bioavailability of pollutants [3].

2.3 Factors affecting Biodegradation of Petroleum Hydrocarbons

The extent of hydrocarbon biodegradation in contaminated soils is critically dependent upon three factors: (a) the creation of optimal environmental conditions to stimulate biodegradative activity, (b) the predominant petroleum hydrocarbon types in the contaminated matrix and (c) the bioavailability of the contaminants to microorganisms.

The composition and inherent biodegradability of the petroleum hydrocarbon pollutant is the first and foremost important consideration when the suitability of a remediation approach is to be assessed. Among physical factors, temperature plays an important role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora. Temperature also affects the solubility of hydrocarbons. Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with the decreasing temperature. Highest degradation rates generally occur in the range $30–40^0$ C in soil environments, $20–30^0$ C in some freshwater environments and $15–20^0$ C in marine environments.

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron. Some of these nutrients

could become limiting factor thus affecting the biodegradation processes. Addition of nutrients is necessary to enhance the biodegradation of oil pollutant. On the other hand, excessive nutrient concentrations can also inhibit the biodegradation activity. Several authors have reported the negative effects of high NPK levels on the biodegradation of hydrocarbons especially on aromatics [3].

The following table summarizes some of the factors that affect the rate of biodegradation.

Table 1: Factors affecting rate of biodegradation [17]

Limiting factor	Explanation or examples
Petroleum hydrocarbon- composition (PHC).	Structure, amount, toxicity.
Physical state	Aggregation, spreading, dispersion, adsorption.
Weathering	Evaporation, photooxidation.
Water potential	Osmotic and matrix forces, exclusion of water from hydrophobic aggregates
Temperature	Influence on evaporation and degradation rates.
Oxidant	O_2 required to initiate oxidation, NO_3^- or SO_4^{2-} to sustain, PHC biodegradation.
Mineral nutrients	N, P, Fe may be limiting
Reaction	Low pH may be limiting
Microorganisms	PHC degraders may be absent or low in numbers

2.4 Literature cited for Soil Methodology

- Sharma and Rehman [18] performed in vitro experiments by taking petrol pump soils and diesel in flasks with micronutrients and macronutrients. Sterlized hydrocarbon contaminated soil was weighed and placed in six different Erlenmeyer flasks. Five different soil samples were prepared to examine the effect of different soil conditions on the degradation of petroleum products. These soils were acidic soil, alkaline soil, soil rich in micronutrients and soil rich in macronutrients. One flask served as control. The flasks were autoclaved and supplemented with hydrocarbons. The bacterial consortium was prepared by inoculating nutrient broth by a loopful of *Moraxella saccharolytica, Alteromonas putrefaciens, Kl. Pnuemoniae subsp. aerogenes* and *Psuedomonas fragi*, which were obtained earlier from diesel contaminated soils. The broth was incubated at 37 deg. And at OD 660 nm was analysed everyday. After

attaining the required absorbency of 1 at 660 nm, 1 ml of broth was taken out and centrifuged. The supernatant was discarded and the pellet was used as bacterial consortium inoculum. The soil adsorbed with hydrocarbon was inoculated with this under sterilized conditions and kept at 26 to 28 deg. The control flask was maintained with diesel and soil without any inoculum and the experiments were carried out for 30 days in triplicates. Diesel from each of the flasks was taken out, centrifuged and analysed using Gas Chromatography. The chromatogram obtained showed various peaks and all were significantly different from control. Dodecane, Pentadecane, Tetradecane were present in soils supplemented with macro- and micronutrients and alkanized soils. Smaller peaks were seen in soils supplemented with beef extract, yeast extract and peptone. It showed that the bacterial consortium degraded more favourably in acidic soil and soil containing beef and yeast extract.

- Mehrsabi et al. [19] investigated biodegradation of petroleum hydrocarbons in 3 different media differing in the kind of petroleum fractions. Soil samples were obtained from 0.5 and 1 m depth as well as from the surface near the contaminated areas close to the Storage of oily products. It was then sieved and made into a solution. An Erlenmeyer Flask was prepared with this solution, mineral salts and synthetic mixture of oil hydrocarbons. It was incubated for 15 days and then transferred to another flask with the same conditions. This was done 4 times so as to increase the colony forming units. Six flasks were then prepared with 200 g dw of soil contaminated with 4g of petroleum. Two flasks had gas oil, two had kerosene and two had a mixture of petroleum fractions. In each of these sets one was poisoned with sodium aside, these did not receive inoculum while the other three did. The ratio of C/N, N/P, and P/K was also maintained by agricultural water soluble inorganic

fertilizer. The pH was adjusted by adding CaO and the flasks were incubated in the dark for 5 months. To avoid anaerobic conditions they were shaken regularly and were taken for experimentation every 15 days. The soil microbial count was done by plate count method and dehydrogenase activity was also monitored. The number of microorganisms, as assessed by plate counts in the soils contaminated with gas oil, kerosene and synthetic mixture at the start of the experiment were 20 × 107, 15 × 107 and 20 × 10^7 cfu/g dw, respectively and at the end of incubation period these numbers were reduced to 10 × 10^7, 2 × 10^7, 0.8 × 10^7 cfu/g dw soil. The numbers of microorganisms were reduced significantly after almost 3 months of incubation. The activities of dehydrogenase enzyme in the non-poisoned flasks after starting the experiment were 120, 100 and 112 μg TPF/g dw.2h soil, respectively. The enzyme activity increased after about 25 days of incubation.

A considerable loss of hydrocarbons was attributed to abiotic processes. Hydrocarbon loss by biological processes was considered to be the difference between the eliminated amounts of hydrocarbons in poisoned control soils and non-poisoned soils. The amount of hydrocarbons lost by biological processes was significant within 5 months. It was shown that the inoculum addition to soil could stimulate bioremediation of the TPH. N-alkenes with the intermediate chain length (C10 - C24) were degraded most rapidly. Short chain alkenes evaporate rapidly as they are toxic to many microorganisms whereas very long chain alkenes are increasingly resistant to biodegradation. In this study, biodegradation of gas oil with intermediate number of carbon was greater than others. Kerosene containing light hydrocarbons showed that its abiotic reduction was greater than gas oil. In mixture, furnace oil containing long chain hydrocarbons showed that its biodegradation was smaller than gas oil. Soil dehydrogenase activity reflected a wide range of microbial oxidative activities.

- Rocha et. al [20] studied the degradation of diesel in soil at laboratory as well as pilot scale experimentation. Biodegradation of diesel oil was performed using a diesel oil-degrading bacterial consortium for both laboratory and pilot scale studies. The bacterial consortium was prepared in liquid in two steps, first in liquid and then in soil. The results showed that the concentration of diesel in the treated soil was reduced to <15% of the initial concentration, within 35 days in both laboratory and pilot scale experiments. As a contrast in the control systems the initial concentration of diesel was reduced by only 5 and 15% in laboratory and pilot scale experiments respectively. Diesel biodegradation rate with the consortium was slightly enhanced by the addition of NH_4NO_3. The enhancement of the microbial activity in hydrocarbon-contaminated soil was inferred to be achieved with the combination of stepwise soil inoculation and nutrient addition.

- Khashayar and Mahsa [21] studied the difference between pure compound enhanced cultures and mixture of fractions. The procedure followed for preparation of different samples was done similarly as done by Mehrsabi et. al [19] except that here there was no poisoning of 3 flasks. The qualitative analysis was carried out using Gas Chromatography and it was found that the culture that was enhanced using a pure compound hydrocarbon could degrade more than 80% of the initial amount present. This was not the case for the culture that was enhanced using a mixture of hydrocarbons like crude oil.

- Tonkova et. al [22] studied the Antarctic soils and their capabilities for degradation of hydrocarbons. Seventeen different isolates were obtained from soil samples of three

regions of Antarctica: Casey Station, Dewart Island and Terra Nova Bay. Most of them were pure aerobic bacteria. They were tested for their ability to grow on mineral salt agar plates which had been supplemented with one of the model *n*-alkanes or aromatic hydrocarbons i.e. hexane, heptane, paraffin, benzene, toluene, naphthalene and kerosene. They were analysed for cell hydrophobicity, the ability to produce anionic glycolipids as well as extracellular emulsifying activity on the basis of growth of soil isolates on hydrocarbons. The results showed many different degraders with a wide range of abilities to grow on both types of hydrocarbons, also good production of glycolipids and emulsifying activity. In conclusion a mixed culture of strains was proposed, which may find application for bioremediation at a moderate temperature of soil environments polluted with different hydrocarbons.

- Roy et. al [23] studied the decomposition of polyaromatic hydrocarbons using mixed culture. Mixed strains were isolated from soil of petrol stations of different Indian cities like Delhi, Hyderabad and Kolkata. These were studied using simulated mixture of anthracene and naphthalene as carbon source in methanol solution. The cell growth curve and substrate depletion time history curve obtained from batch fermentative process showed that the reaction engineering behaviour of these microbiological systems could be represented by classical substrate uninhibited Monod's model. The intrinsic kinetic parameters μ_{max} and K_S were evaluated following differential analysis of experimental data.

- Benyahia et. al [24] studied the ex-situ method of biodegradation by applying the bioaugmentation approach. Their experiment involved four samples of soil; clean soil, artificially contaminated by crude oil, crude oil contaminated with added commercial

bacterial product and crude oil contaminated with Hg_2Cl_2 soils which were analysed for Total Petroleum Hydrocarbon (TPH), Polycyclic Aromatic Hydrocarbon(PAH), Total Organic Carbon(TOC), polychlorinated biphenyls(PCB) and elemental analysis(H,S,N,P) before and after the bioremediation treatment. Their results showed the adaptation period, maximum oil degrading period and a decaying period. They also showed identical trends for indigenous and commercially added bacteria and that simpler and bioavailable crude oils degrade first. The study showed that by adding bacteria the respiration rates increased by a large margin which again highlights the benefits of bioaugmentation. Also that if macro and micro nutrients are not added then the bacteria metabolize the hydrocarbons into CO_2 and water to a greater extent. It sheds light on how the biopile system can be advantageous and can replace the land farming practice.

- Liu et. al [25] studied the in-situ bioremediation by biostimulation for 360 days. Two plots of soil were considered-treated and control. 5% manure was added to the treated plot and its effect was studied. The sampling was done at 0 and 360 days. The physicochemical properties from each subplot were characterized and the TPH was measured. Soil microbial counts and community level physiological profiling were examined. Also a biotoxicity assay was carried out. The results showed that the soil that was initially black because of contamination was thinly scattered with wild plants after the bioremediation. Also due to the addition of manure the N, P, K levels increased which again stimulated the growth of hydrocarbon degraders. The study also showed that different components of hydrocarbons had different degrees of degradability. The TPH content in the treated plot reduced by 58.2% where as in the control plot it reduced by 15.6%. It was hence inferred that the levels of nitrogen and

other components wasn't enough in the soil to promote the growth of indigenous degraders but with addition of these, in the form of manure, the microbial activity and diversity increased after bioremediation for 1 year. The levels of TPH were still quite high and further treatment was necessary.

- Atlas [26] presented a review paper that explored the chemistry of petroleum biodegradation-degradation of individual hydrocarbons and within the petroleum, the taxonomic relationships of hydrocarbon utilizing microorganisms, the distribution of hydrocarbon utilizing microorganisms, environmental factors affecting biodegradation, the physical state of oil pollutants and a few case histories.

  He has reviewed the metabolic pathways followed by n-alkanes, branched isoprenoid alkanes, cycloalkanes which were found to be resistant to microbial attack, aromatic hydrocarbons and asphaltic components of the oil during the course of biodegradation. The potential activities of the hydrocarbon degrading bacteria using ^{14}C radiolabelled hydrocarbons have also been reported. The order of biodegradation potential has been reported as hexadecane>naphthalene>>pristane>benzanthracene. The author has also reported the findings of different authors in terms of genera of fungi, bacteria, algae, yeast etc. that are capable of biodegradation in various environments. The factors that affect biodegradation are also reported. Temperature has a very wide range for the microbes to degrade but higher the temperature faster is the degradation. Nutrients such as Carbon, Phosphorous, Nitrogen and nutrient ratios such as C/N, C/P are reported to be important considerations for biodegradation. Oxygen is another important parameter; anaerobic biodegradation is shown to give negligible results. In conclusion it is said that the interactive nature among the microorganisms, oil and environment is not understood completely and that more

research is required to improve the predictive nature of the fate of oil pollutants and to develop models for that.

- Brackstad and Bonaunet[27] studied the biodegradation potential of marine microorganisms in seawater at temperatures 0 and 5 degree Celsius. The experiments were conducted with crude mineral oil immobilized as thin film on hydrophobic adsorbent in sterile seawater. Chemical and respirometric analysis showed that the degradation of small aromatic compounds was faster than that of larger PAHs or higher alkanes. The degradation of n-hexadecane was substantial at both 0 and 5 degree Celsius but was delayed at 0 degree. The results also showed that the degradation of some other components may not be as substantial at lower temperatures. Thus it was inferred that marine microbial communities from cold seawater do have the potential for biodegradation at temperature below 5 degrees Celsius.

- Atlas [28] in this paper has studied the bioremediation of contaminated groundwater. He has also discussed how the populations of indigenous microorganisms and physiological capabilities of these affect the degradation of petroleum hydrocarbons. He has also discussed the genetic changes that occur in microorganisms in such an environment. Microorganisms in the contaminated areas adapt according to the environment as a result of which genetic mutations are caused in the subsequent generations causing them to become hydrocarbon degraders. In normal conditions the degraders are less than 1% of the population but in contaminated areas they are 1% to 10% of the population. Also these adapted microorganisms can react to contamination within hours. He has reviewed the time frame required for bioremediation which is

studied by different authors by adopting practices like forced aeration and Nitrogen or phosphorous addition. He has reviewed the feasibility of bioremediation in case of marine oil spill by nutrient augmentation. The possibility of using genetically modified microbes for bioremediation is also discussed; seeding with such microbes can preclude the competition between different strains in a mixed culture. But the success of this process is still ambiguous and requires more research.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

The experiment involves the following steps:

1. Collection of soil from an area where garages discharge their oil waste.
2. Isolation of microorganism from the collected soil sample.
3. Optimization of process parameters using octane as a carbon source.
4. Degradation study using heavy and light crude oil as the carbon source.

3.1 Isolation of microorganism

A solution of 500 ml water, 5.25 grams of M9 salts, 5 ml of engine oil(1%) and 5% of soil is made and left alone for 4 months in a beaker. At the end of 4 months some fungal growth is observed. It is identified as fungal growth because of its mycelia. 1 ml of this contaminated water from the white zone is then taken and series dilution of the order 10^{-6} is carried out as the concentration in the contaminated water is too high. The solution from the last dilution is then taken and streaked on a nutrient agar medium where glucose acts as the carbon source. This culture is incubated at a temperature of 28 degree Celsius and 7.4 pH for 10 days. Fungal colonies can be seen at the end of 10 days. Again fungal colonies are identified by their mycelia.

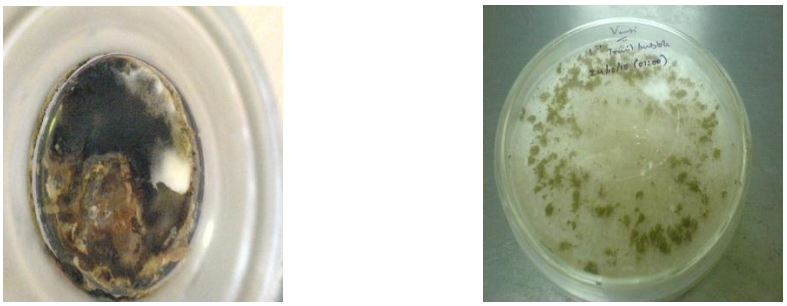

Figure 3: Culture after (a) 4 months (b) series dilution

3.2 Optimization of Process Parameters

It is evident from the literature review that the factors affecting the process of biodegradation are temperature, pH and nutrients. Nutrients required for growth are Nitrogen, Phosphorous, Carbon, Magnesium, Sulphur and trace elements which include calcium and iron. Phosphorous is present in the nutrient medium that is prepared for the cultures and hence cannot be optimized. Similarly sulphur cannot be optimized because it is present in the magnesium salt as sulphate. The rest of the parameters need to be optimized so that maximum growth of the fungus is seen. The growth is analysed in terms of biomass. Also the nitrogen source needs to be optimized first; the source that shows the maximum growth will be used for the optimization experiments.

3.2.1 Nitrogen Source optimization:

The sources considered for optimization are Urea, Peptone, Ammonium chloride, Ammonium Sulphate. The culture is prepared in 4 conical flasks, each with different nitrogen source. In this experiment n-octane is used as a carbon source. The concentration of the broth for one flask or 50 ml of solution is as follows

Table 2: Concentration of Broth

n-Octane	0.5 ml (1%)
Nitrogen Source	0.125g (0.25%)
Magnesium ($MgSO_4.7H_2O$)	0.02g (0.04%)
Potassium Phosphate (KH_2PO_4)	0.05g (0.1%)
Di-potassium phosphate (K_2HPO_4)	0.05g (0.1%)
Trace Elements solution (Content L^{-1}: $CaCl_2.2H_2O$ and $FeSO_4.7H_2O$: 1g)	0.05ml

The flasks and the broth are properly sterilized at 15 psi, and the pH of the broth is measured prior to inoculation with approximately 1ml of the liquid culture of the isolated fungi. The flasks are then sealed with a film of paraffin wax and placed in a shaker at 28 degrees Celsius and 120 rpm for 72 hours; at the end of which the biomass in different flasks is measured in terms of dry weight.

3.2.2 Temperature optimization

The temperatures considered for optimization are 20, 25, 30, 35 and 40 degrees Celsius. The culture is prepared in 5 different flasks, each to be maintained at a different temperature. As it wasn't possible to maintain 5 different temperatures at once, this experiment was carried out in 3 sets. In the first set 20 and 30 degree Celsius temperatures were maintained. In the second set 25 and 35 degree Celsius were maintained and in the third set 40 degree Celsius was maintained. The concentration of the broth was the same as in nitrogen source optimization, but the carbon source used was n-octane. The initial pH and dissolved oxygen (DO) are measured and then the flasks with the broth are sterilized and inoculated. The flasks are then sealed and placed in a shaker at 120 rpm and left to for 4 to 5 days, depending on the growth of the fungus.

3.2.3 pH optimization

The pH considered for optimization is 6, 7, 8 and 9. The culture is prepared in 4 different flasks, each to be maintained at a different pH. The concentration of the broth and the carbon source is the same as in temperature optimization. The initial pH and DO measurements are carried out. The different pH are adjusted by the addition of NaCl or HCl solution, a standard 1N solution of the two is prepared in order to adjust the pH. The flasks are sterilized, inoculated, sealed and placed at 120 rpm and the temperature obtained as optimum from the

previous experiment. It is left to grow for 4 to 5 days, but the pH is measured and re-adjusted if required at an interval of 6 hours for the entire growth period.

3.2.4 Nitrogen Concentration optimization

The different concentrations considered for optimization are 0.25g (0.5%), 0.5g(1%), 0.75g(15%) and 1g(2%), 1.25g (2.5%). The culture is again prepared in 5 different flasks each to be added with different amount of nitrogen source. The concentration of the broth and the carbon source are the same. The initial pH and DO measurements are carried out. The flasks are sterilized, inoculated, sealed and placed at the optimum pH, optimum temperature and 120 rpm. It is left to grow for 4 to 5 days.

3.2.5 Magnesium Concentration optimization

The different concentrations considered for magnesium are 0.01g, 0.02g, 0.04g, 0.06g and 0.08g. The culture is prepared in 5 different flasks such that each flask has a different concentration of magnesium. The concentration of the broth and the carbon source are the same except that the concentration of nitrogen source is the one obtained after optimization. The initial pH and DO measurements are carried out. The flasks are sterilized, inoculated, sealed and placed at the optimum pH, optimum temperature and 120 rpm. It is left to grow for 4 to 5 days.

3.2.6 Trace Elements Optimization

The different concentrations considered for magnesium are 0.005ml, 0.01ml, 0.02ml, 0.03ml and0.04ml. The culture is prepared in 5 different flasks such that each flask has a different concentration of trace elements. The concentration of the broth and the carbon source are the same except that the concentrations of nitrogen source and magnesium are the ones obtained after optimization. The initial pH and DO measurements are carried out. The flasks are

sterilized, inoculated, sealed and placed at the optimum pH, optimum temperature and 120 rpm. It is left to grow for 4 to 5 days.

3.2.7 Analysis

The analysis is carried out in terms of biomass. As it is a liquid culture colonies cannot be seen, also photospectrometer cannot be used as the mycelia interfere with the waves. Thus dry weight analysis is carried out. In this method a filter paper (Whatman No. 1) is initially weighed and then used to filter the entire culture broth present in a flask. This filter paper now contains the colonies of fungi that are formed in the culture. It is dried at 45^0 C for 12 hours after which it is weighed again. The difference of weight of the filter paper gives the amount of biomass that has grown. The final pH and DO values are also measured.

3.3 Degradation Study

For studying the degradation of crude oil the carbon source used is heavy and light crude oil separately. In this study the carbon concentration is varied in terms of crude oil concentration and the number of days for which the degradation takes place is also varied. The concentrations considered are 1g, 5g, 10g and 15g. For the experiment of number of days 5g crude oil is added and left for 15, 30 and 45 days separately. The culture was prepared in 20 different flasks. In 4 of the flasks the concentration of light crude oil was varied i.e. 1g, 5g, 10g and 15g. Similarly in another 4 flasks the concentration of heavy crude oil and was varied. In another 6 flasks the amount of carbon source was constant but they were to be kept for different number of days and hence had to be analysed at different times. 3 of them had light crude oil as the carbon source and 3 of them had heavy crude oil as the carbon source. The remaining 6 flasks acted as control, 3 each for 15, 30, and 45 days for both light and heavy crude oil. The concentration of the broth is the same as in previous cases but the temperature, nitrogen concentration, pH, magnesium and trace elements concentration are the

ones that have been obtained as results in the optimization study. The initial pH and DO are measured. The flasks are sterilized (before addition of crude oil), inoculated, sealed and placed in a shaker at 110 rpm. At first they are monitored after a week but following the growth they are monitored every 2 to 3 days.

For the analysis of the amount of oil degraded the procedure followed is as follows:

Note the initial weight of the filter paper. Filter the solution in the degradation flask. Scratch the attached fungal biomass to the conical flask walls using a spatula. Add 5 ml of hexane in to the flask & stir to remove any oil leftover in the flask. The hexane present in the flask is transferred into a beaker (initial weight is known) through the filter paper containing oil & fungal biomass. Add 10 ml of hexane in to the filter paper to wash the oil leftover in the paper, the hexane is collected in to the beaker in which first set of hexane wash is collected. Note the weight of hexane containing oil. As the initial weight of the hexane solvent is measured before using, we can deduct the final weight of the hexane with the initial weight of the hexane to get the oil weight. By this we can find the % of degraded. Now the filter paper with fungal biomass is dried at 45˚C in hot air oven for 24 hours to get the dry bio mass.

The list of equipment used in the process is as follows:

Table 3: List of Equipment

Instruments	Make	Function
Vertical Autoclave	Test Master	Sterilization
Analytical Balance	Sartorius	Weight Measurement
Laminar airflow	Zhichen (ZhJH-1109C)	Aseptic Environment
pH	EuTech Instruments	Measurement of pH
Incubator shaker	Environmental Orbital Shaker	Shaking and Temperature Control
DO meter	Hach (HQ10)	Measurement of DO

CHAPTER 4

RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Optimization Results:

4.1.1 Nitrogen source optimization

The results for nitrogen source optimization are plotted on a pie chart as follows:

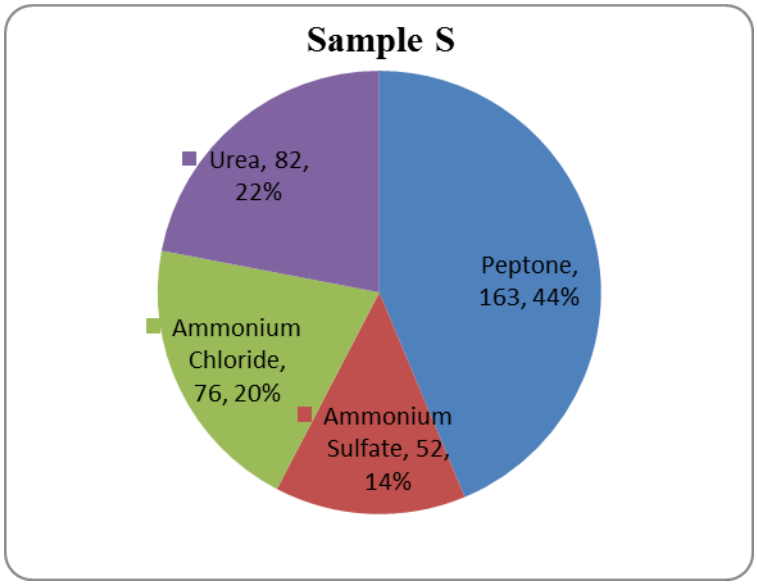

Figure 4: Nitrogen Source Pie Diagram

The biomass obtained is also shown on the chart. It is evident that peptone is the most favourable nitrogen source as it gives the highest amount of biomass but peptone as a source for a number of experiments is not economically favourable. Hence we choose the next best source which is Urea. For all the following experiments for optimization urea is used as the source of nitrogen.

4.1.2 Temperature Optimization

The results for temperature optimization are plotted in the form of a graph as follows:

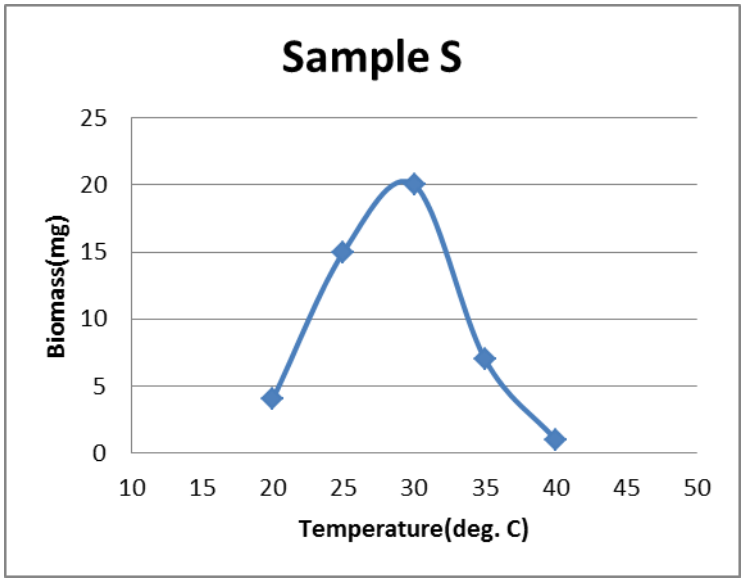

Table 4: Temperature Results

Temperature	Biomass(mg)
20	4
25	15
30	20
35	7
40	1

Figure 5: Biomass vs Temperature

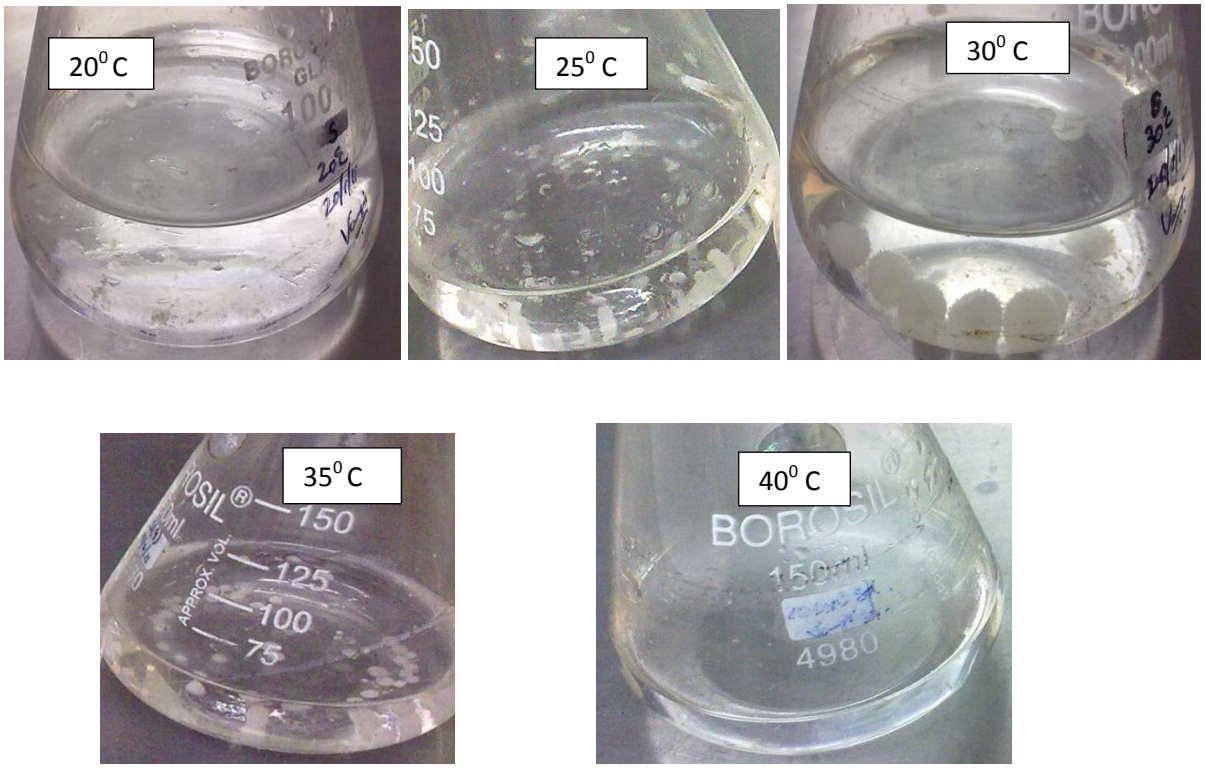

Figure 6: Temperature Optimization Pictures

It is evident from the graph that growth is maximum at 30^0. There was very little growth at 20^0 and it increased with increasing temperature. But after 30^0 it was seen that the fungus had started degenerating and at 40^0 there was no growth at all. Hence it can be inferred that at temperatures as high as 40^0 this particular fungus does not grow favourably. Thus the desired temperature for growth is between 28^0 to 30^0.

4.1.3 pH optimization

The results for pH optimization are as follows:

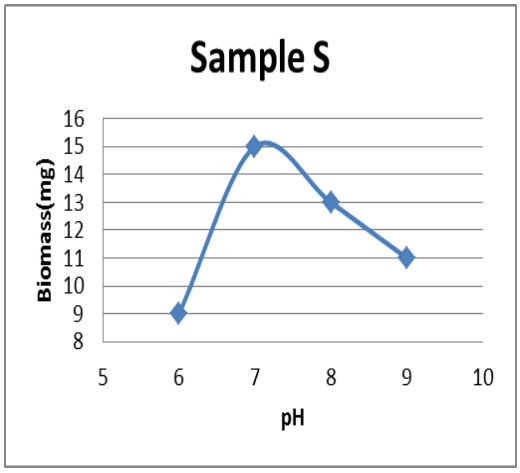

Figure 7: Biomass vs pH

Table 5: pH Optimization Results

pH	Biomass(mg)
6	9
7	15
8	13
9	11

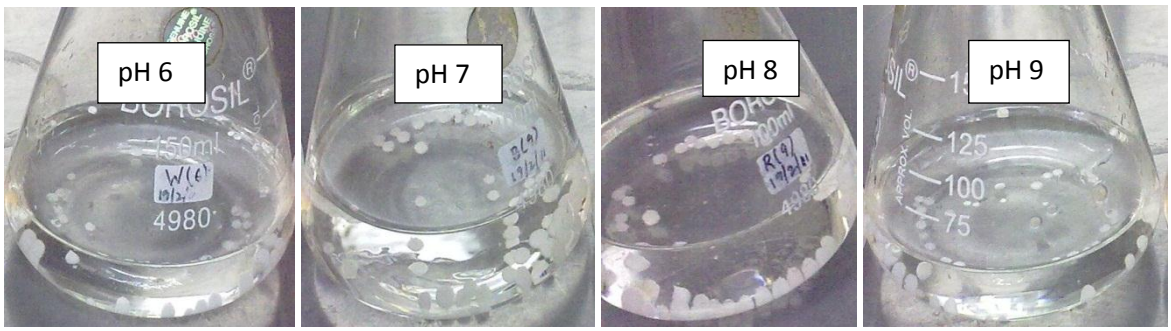

Figure 8: pH Optimization Pictures

Maximum growth can be seen around pH 7. The growth increases with the increasing pH but after 7.5 it starts declining. This could be indicative of the fact that higher pH or lower acid

nature can prove to be denaturing for the fungus. Thus it can be inferred that the most optimum pH range is 7 to 7.5.

4.1.4 Nitrogen Concentration optimization

The results for the nitrogen concentration optimization are as follows:

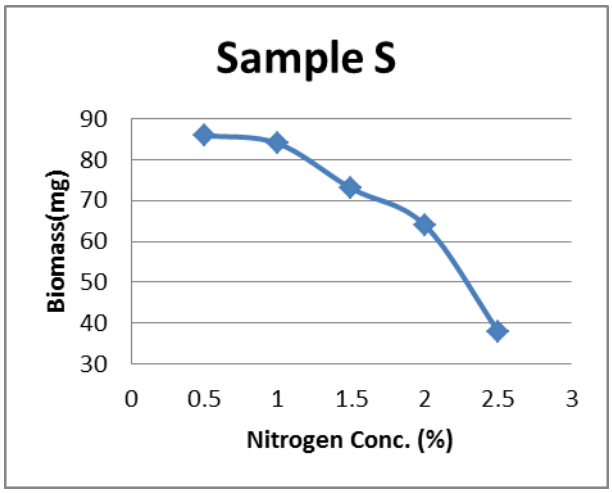

Table 6: Nitrogen Conc. Optimization Results

N_2 Conc.(%)	Biomass(mg)
0.5	86
1	84
1.5	73
2	64
2.5	38

Figure 9: Biomass vs Nitrogen Conc.

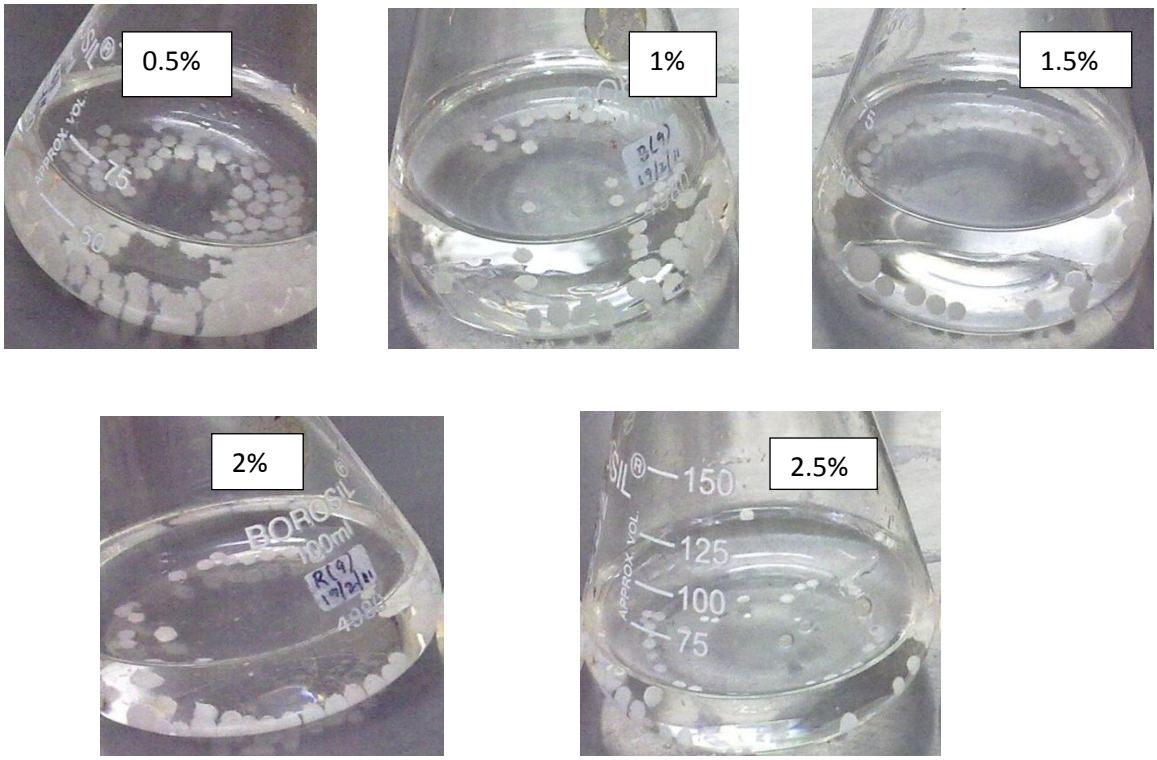

Figure 10: Nitrogen Optimization Pictures

It is evident that maximum growth is seen at a concentration of 0.5% or 0.25g. With increasing concentration of nitrogen the fungus is getting saturated and hence starts showing degeneration. The optimum concentration hence is inferred to be 0.5% or 0.25g in 50 ml solution.

4.1.5 Magnesium Concentration optimization

The results obtained can be shown as follows:

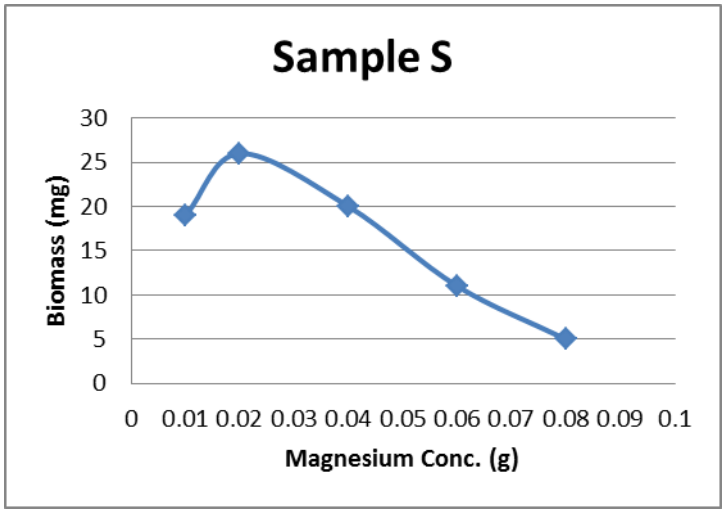

Table 7: Magnesium Conc. Results

Magnesium conc.(g)	Biomass(mg)
0.01	19
0.02	26
0.04	20
0.06	11
0.08	5

Figure 11: Biomass vs Magnesium Conc.

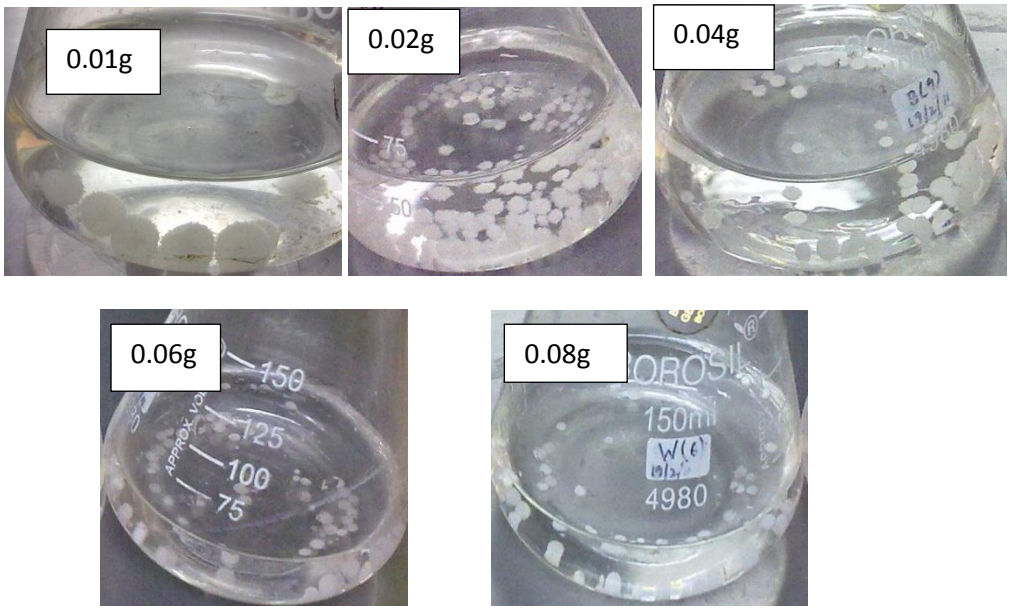

Figure 12: Magnesium Optimization Pictures

It is evident form the pictures that the maximum growth is at 0.02g concentration of magnesium. Again as in Nitrogen concentration optimization, with increasing concentration of nutrients the growth is decreasing. This is indicative of the fact that even excess of nutrients can cause denaturing of the fungus. Thus it is important to add the nutrients in the correct amount. Hence the optimum concentration of Magnesium is 0.02g in 50 ml solution.

4.1.6 Trace Elements Concentration optimization

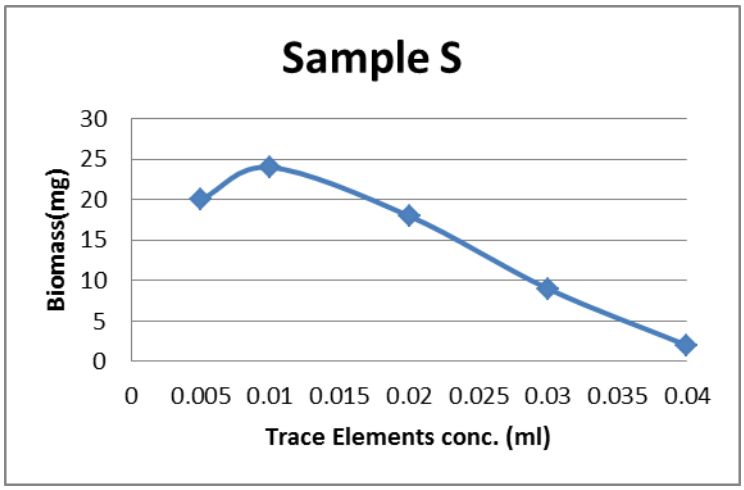

Table 8: Trace Elements Optimization Results

Trace Elements conc.(ml)	Biomass(mg)
0.005	20
0.01	24
0.02	18
0.03	9
0.04	2

Figure 13: Biomass vs Trace Elements Conc.

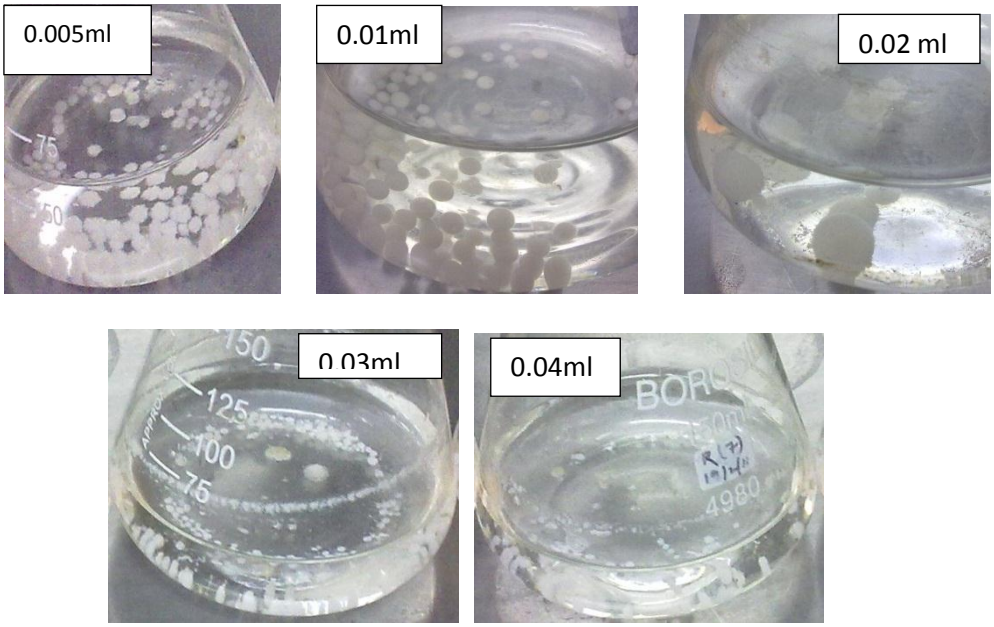

Figure 14: Trace Elements Conc. Optimization Pictures

It is evident from the graph and pictures that the maximum growth is seen at 0.02% (0.01ml) concentration of trace elements. As seen with nitrogen at high concentrations the fungus starts degenerating. It is hence important to maintain the right balance of nutrients for proper growth.

4.2 Degradation Study

The results after 45 days of degradation study for light crude oil are as follows:

Table 9: Light Crude Oil Degradation Study Results

S. No	Sample Name	Initial Oil (g)	Final Oil (g)	Degradation %	Biomass (mg)	pH	D.O
1.	**1 L – 20 Days**	1	0.22	78	48.1	8.91	4.62
2.	**5 L – 15 Days**	5	2.9	42	53.9	8.92	5.08
3.	**5 L – 30 Days**	5	2.1	58	73.6	8.65	4.62
4.	**5 L – 45 Days**	5	1.48	70.4	62.8	9.26	3.18
5.	**10 L – 30 Days**	10	8.25	17.5	15.5	8.41	5.11
6.	**15 L – 30 Days**	15	14.17	5.53	6	7.89	5.81

Table 10: Light Crude Oil Degradation Study Results

Before Degradation

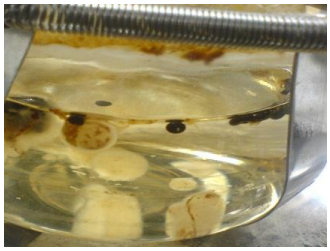
After Degradation

Figure 15: Pictures for light crude oil degradation study

These results can be shown graphically as:

Initial Concentration vs Final Concentration:

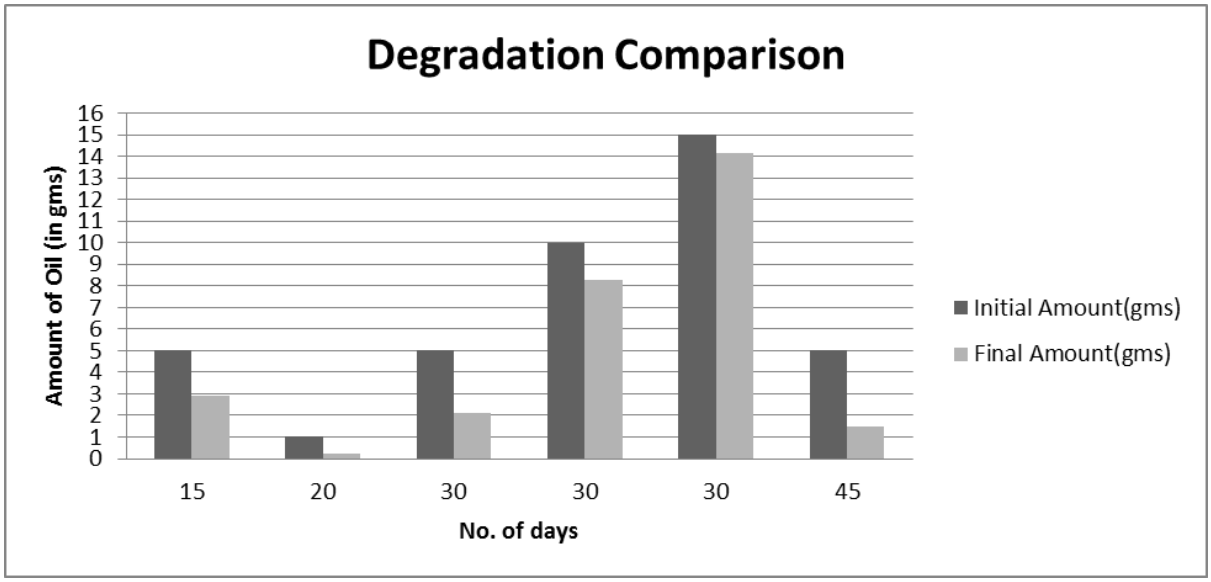

Figure 16: Initial Amount vs Final Amount(light Crude Oil)

Biomass vs No. of days and initial amount added:

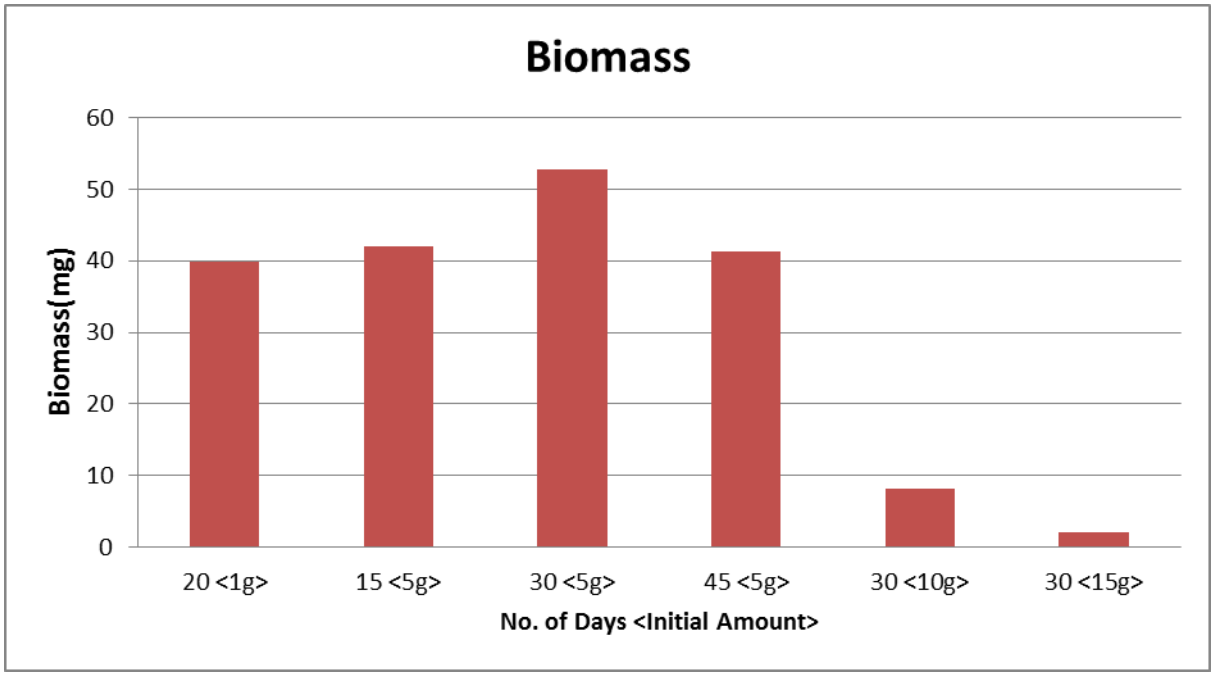

Figure 17: Biomass vs No. of Days(Light Crude Oil)

Dissolved Oxygen vs No. of days and amount of oil added:

The initial value of DO was measured to be 5.7ppm.

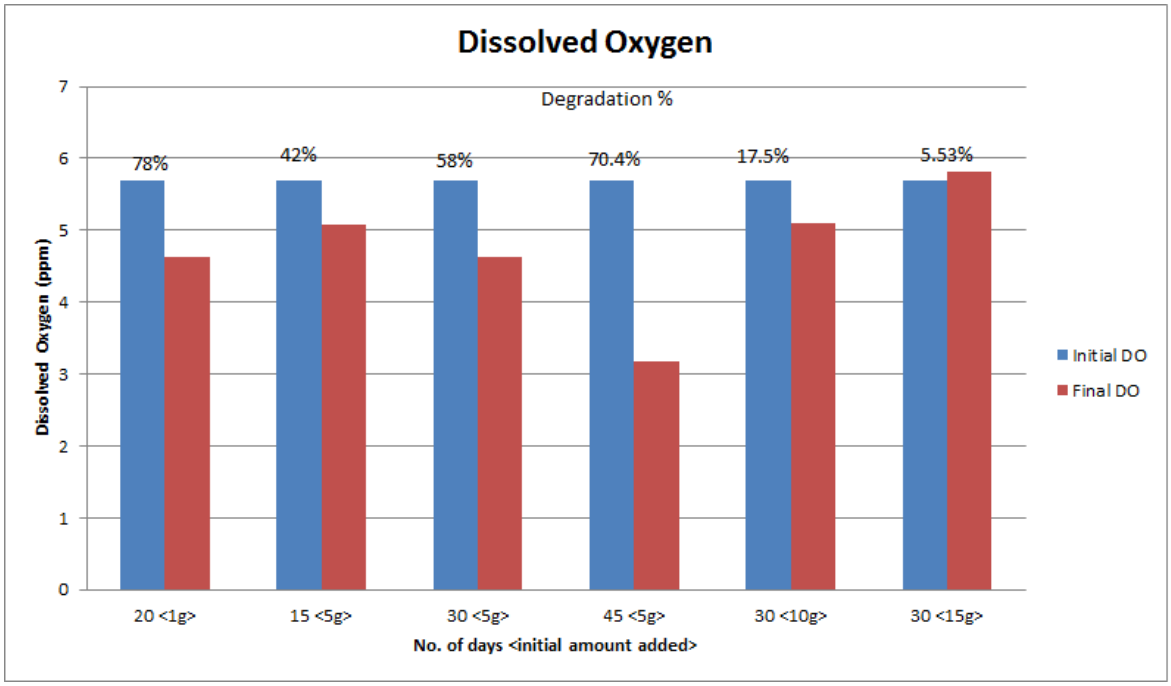

Figure 18: Dissolved Oxygen vs No. of Days (Light Crude oil)

The results for heavy crude oil as carbon source are as follows:

Table 11: Degradation Study Results for Heavy Crude Oil

S. No	Sample Name	Initial Oil (g)	Final Oil (g)	Degradation %	Biomass (mg)	pH	D.O
1.	**1 H – 20 Days**	1	0. 58	42	39.8	8.85	5.09
2.	**5 H – 15 Days**	5	3.28	10.34	42.1	8.78	5.61
3.	**5 H – 30 Days**	5	2.7	46	52.8	8.71	4.88
4.	**5 H – 45 Days**	5	2.18	56.4	41.3	8.78	3.61
5.	**10 H – 30 Days**	10	9.75	2.5	8.22	8.23	5.61
6.	**15 H – 30 Days**	15	14.81	1.26	2	7.78	5.89

Before Degradation


After Degradation

Figure 19: Pictures for Heavy Crude Oil Degradation Study

These results can be shown graphically as follows:

Initial concentration vs final concentration:

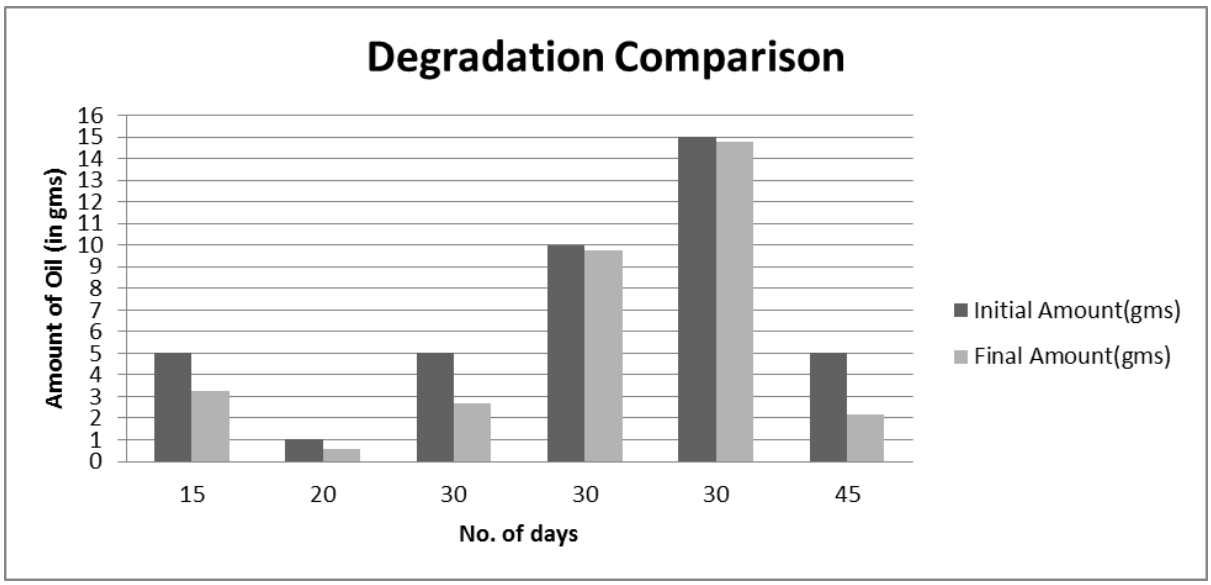

Figure 20: Initial Amount vs Final Amount(Heavy Crude Oil)

Dissolved Oxygen vs No. of days.

The initial amount of DO was 5.7 ppm.

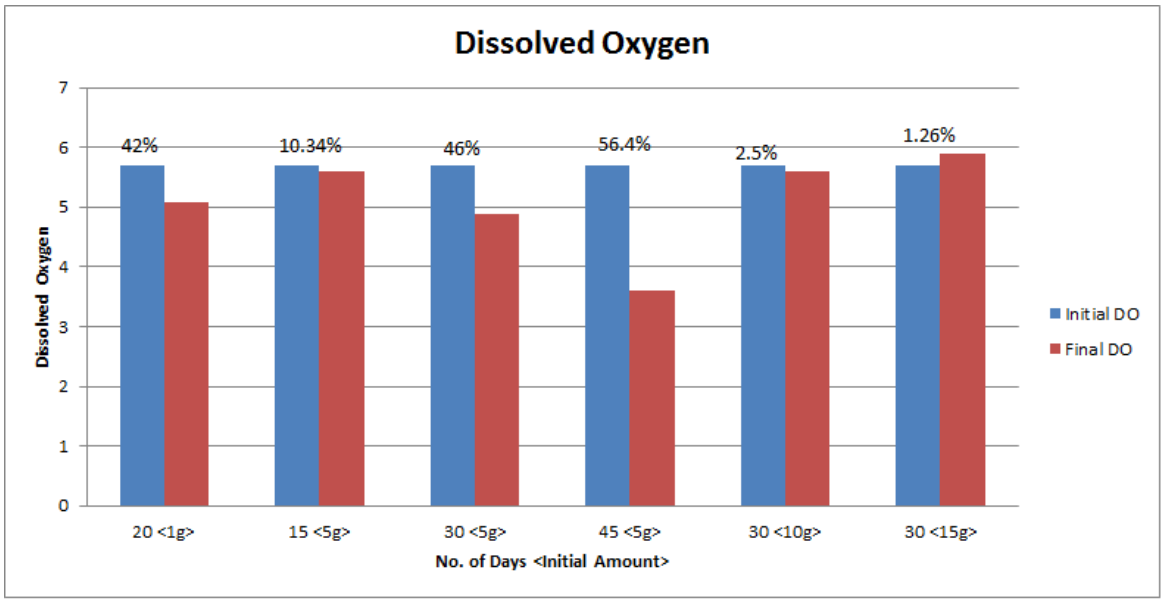

Figure 21: Dissolved Oxygen vs No. of days(Heavy Crude Oil)

Biomass vs No. of days and initial amount added

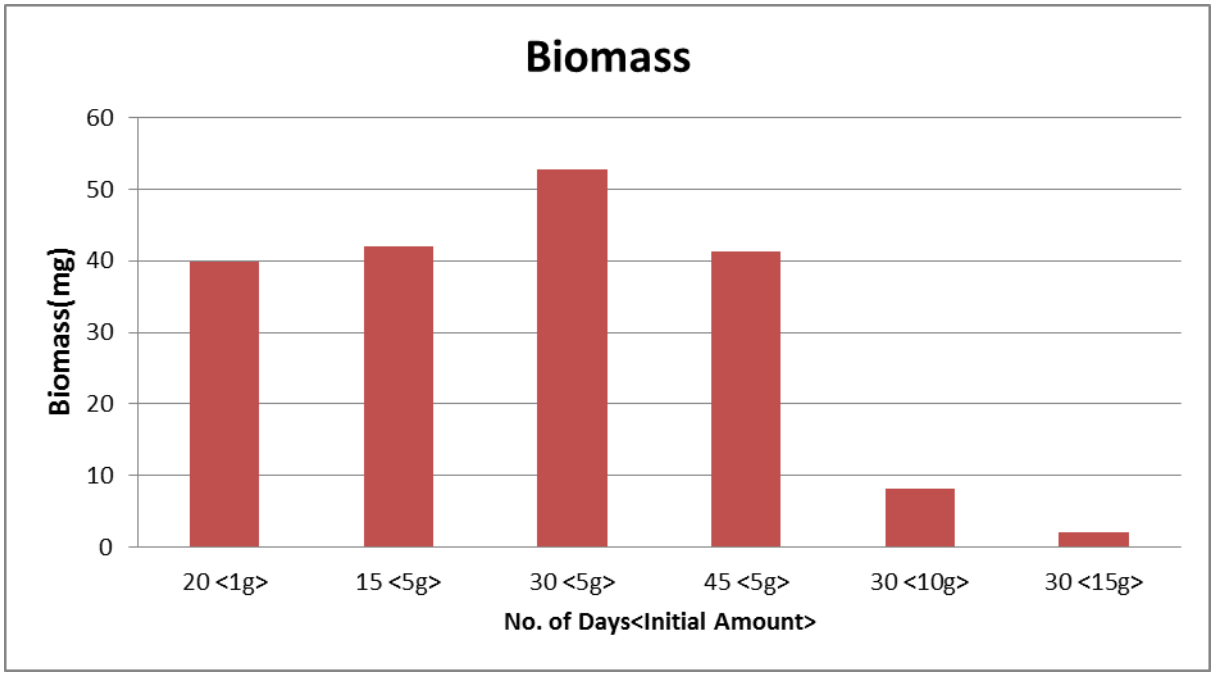

Figure 22: Biomass vs No. of Days(Heavy Crude oil)

Light crude oil contains components with 1 to 10 carbon atoms. They have small molecules with less number of atoms in each molecule. As a result they have high volatility and can be evaporated or dissolved very easily. It poses a greater threat because it can spread very rapidly and penetrate the porous media; also it is potentially flammable and can be easily inhaled by marine or human life. But on the plus side it is more bioavailable than heavy crude oil. It is also evident that light crude oil is degraded almost completely by the fungus(Figure 14, Figure 18). Even with increasing concentration of the oil the fungus is capable of degrading it as is evident form(Figure 15). When the initial amount of oil added is increased to 5g the, significant degradation is still seen at the end of 15, 30 and 45 days; it is almost uniform. The amount of biomass produced during this period is also on the rise and at the same time the dissolved oxygen is on the decline, because of the simple fact that as no external oxygen is provided the biomass is using up the dissolved oxygen for its growth. It can be argued that the highest amount of oxygen used is for the flask placed for 45 days and yet it does not give the highest percentage of degradation, which is given by the flask placed for 20 days; this can be accounted by the fact that the greater the number of days the higher amount of oxygen is required for survival. At the end of 20 days the fungus produces less biomass and hence uses less oxygen but as the amount of oil added was just 1g it could easily degrade it with that much amount of activity; whereas when it is placed for 45 days, firstly the fungus survives for a longer period and hence uses up more oxygen, secondly the initial amount of oil added also is greater by 4g and hence higher amount of activity is needed for degradation, hence the percentage of degradation is relatively less. Another trend that is observed is that when the concentration of oil is increased to as high as 10g, the microbial activity declines drastically shown by the decreased percentage of degradation(Figure 17), decreased production of biomass and very less use of dissolved oxygen(Figure 17). It can be inferred from this result that high concentration of oil is toxic for the fungus and it starts

degenerating. With the concentration increased to 15g the degradation is almost non-existent with increased levels of dissolved oxygen than initial measurement. This may be attributed to the shaking of the culture which causes the DO to increase slightly, and as there are no organisms to use it up the level is seen to rise.

Heavy crude oil contains components that have more than 23 carbon atoms and hence are very bulky molecules which do not undergo evaporation or dissolution. It has a longer residence time and hence the risk of exposure is increased. The toxicity of this oil is low as it does not penetrate through the porous media, but can have a chronic effect if there are residues in the water column; also it is harder to clean-up because of its high density and viscosity. The overall degradation efficiency of the fungus decreases when compare to light crude oil as the maximum degradation achieved for heavy crude oil is 56.4%. The general trends of degradation as observed in light crude oil are seen here too; with increasing amount of oil added the degradation is seen but at highly elevated concentrations it is virtually non-existent. The highest degradation is seen in the flask placed for 45 days with 5g of oil added initially; it can hence be pointed out that the fungus has the capability to degrade even heavy oil but requires a longer duration for it. The degradation is seen to slow down as more and more oxygen is used up in subsequent days. It can be argued that the maximum biomass is produced in the flask placed for 30 days with 5g of initial oil added but it does not give the maximum degradation; as the number of days increase the biomass produced is decreasing which tells us that the denaturation of the fungus has started. At the end of 30 days the biomass produced is more but maybe because of the competition to survive, it degrades less oil or that in the process of degrading the fungus enters the death phase because at 45 days the degradation percentage is maximum. Again as the concentration is increased to 10g or more than that the degradation percentage declines drastically, producing less biomass and

the dissolved oxygen levels are elevated rather than declining. The reason for this is the same as in the case of light crude oil.

Another inference that can be drawn from these experiments is that this fungus is a de-emulsifier. The solution after degradation or even during the process is clear; the oil is trapped inside the mycelia of the fungus leaving the solution clear. Generally oil degrading microorganisms tend to produce surfactants that tend to emulsify the oil and spread it throughout the solution making it murky or brown in colour. The absence of such colouration proves that this fungus does not produce enough surfactants to emulsify the oil or that it produces de-emulsifiers that cause the fungus to entrap the oil rather than spread it throughout.

CHAPTER 5

CONCLUSION

Conclusion

The fungus isolated from the soil sample shows enormous capability to degrade oil and hence can be used as a bioremediation tool for the de-contamination of the water systems. During optimization studies it is found that the optimum temperature for this strain is around 30^0 C and the optimum pH is around 7. This implies that this type of fungus is more suitable to be used in river water systems or the soil around it because the pH of seawater is nearly 9 and hence the fungus may not show its highest potential. The optimum concentration of nitrogen is 0.5% i.e. 250 mg, of magnesium is 0.04% i.e. 20mg, and of trace elements is 0.01ml in 50 ml solution. Only if these conditions are maintained maximum growth of the fungus can be seen and its highest potential of degradation can be tapped.

The degradation study helps us conclude that the strain can degrade both light and heavy crude oil over a range of concentration but not at very high ones. This broadens the spectrum of contaminants that this strain can degrade and hence increases its applicability. Dissolved oxygen is a necessity for this strain to grow or else oxygen will have to be supplied externally as it is one of the major components required for growth. The study shows that it can produce an average biomass of approximately 60 mg which requires approximately 4 to 5 ppm of dissolved oxygen giving an efficiency of approximately 65% degradation considering both light and heavy crude oil over a period of 30 to 45 days. The degradation efficiency is more for light crude oil than its heavier counterpart but we can also conclude that given a longer duration, it can also degrade the heavy crude oil with significant efficiency. The exact strain is yet to be identified but it can be safely considered as viable for clean-up procedures of river water systems.

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